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RESEARCH ARTICLE

The sexual dimorphic inguinal glands of the frog species *Ololygon centralis* (Anura: Hylidae) at light and transmission electron microscopy

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ABSTRACT. The anuran skin characteristically has different types of glands, most of which are microscopic and are spread throughout the skin. Some species have specialized regions where glands agglomerate, forming macroglands. The description of the external morphology of *Ololygon centralis* (Pombal & Bastos, 1996) revealed the presence of an inguinal gland. *Ololygon centralis* is the only species of the genus that has a macrogland. The present study found these inguinal macroglands to be present only on male specimens, thus characterizing it as a sexually dimorphic skin gland. Microscopic analysis revealed that these glands are composed of many syncytial units involved by myoepithelial cells. The center of the syncytium is full of a proteinaceous secretion with a basic pH and the absence of sugar residues. Similar glands observed in other anuran species have been associated with pheromone production, suggesting that the inguinal glands described for *O. centralis* males may have a similar function.

KEY WORDS. Histochemistry, morphology, skin, serous gland.

INTRODUCTION

The skin of anurans characteristically has different types of glands, the secretions of which are responsible for a variety of different functions including poison production, antimicrobial protection and the maintenance of skin moisture (Duellman and Trueb 1994, Azevedo et al. 2005, Felsemburgh et al. 2009). The most common types of anuran glands are mucous glands, which mainly secrete glycoproteins, and granular (poison) glands (Alvarez et al. 2005, Lenzi-Matos et al. 2005, Felsemburgh et al. 2007, Rigolo et al. 2008, Jared et al. 2009, Felsemburgh et al. 2009, Brunetti et al. 2012). Granular glands are composed of a syncytial structure that secretes variable protein products and depending on the species, also produce amines, alkaloids, and bufadienolides (Duellman and Trueb 1994, Sciani et al. 2013). Some species, particularly some arboreal species, possess lipid glands, which seem to function in the prevention of water loss (Lacombe et al. 2000, Felsemburgh et al. 2007).

Most anuran skin glands are distributed throughout the body surface with some differences in distribution between dorsal, ventral and lateral regions. Some species have specialized regions with large glandular acini agglomerates that form skin protuberances. These macroglands are named according to their location on the body, such as parotoid, inguinal and lumbar glands. Macroglands usually produce toxins that are used in active or passive defense (Jared et al. 2009, Antoniazzi et al. 2013,

Mailho-Fontana et al. 2014). Some species have sexually dimorphic skin glands (SDSGs), which are involved in reproduction, such as in *Boana punctata* Schneider, 1799 (Brunetti et al. 2012).

The skin glands of at least 12 species of the related genera *Scinax* Wagler, 1830 and *Ololygon* Fitzinger, 1843 were investigated (Terreni et al. 2002, Silva et al. 2017). All these species have both mucous and granular glands distributed in their skin. Only four of these species have clusters of granular glands: *O. angrensis* Lutz, 1973, *O. flavoguttata* Lutz & Lutz, 1939, *O. albicans* Bokermann, 1967 and *S. hayii* Barbour, 1909 (Silva et al. 2017) – but with no skin protuberances typical of macroglands.

The description of the external morphology of *Ololygon centralis* (Pombal & Bastos, 1996) revealed a sizable inguinal gland (Pombal and Bastos 1996). This species was initially placed *Scinax*, and grouped in the *Scinax catharinae* Boulenguer, 1888, species group. Later, using a phylogeographic approach, Duellman et al. (2016) suggested that all the species included in the *S. catharinae* group should be placed in *Ololygon*.

Ololygon centralis has a small body with the inguinal region and parts of the thighs being yellowish and dark brownish, where it is possible to find the well distinct glands in males (Pombal and Bastos 1996). This species occurs mainly in or near gallery forest of the Brazilian Cerrado (Frost 2018) and is one of the few species of the genus to occur in this biome (Pombal and Bastos 1996, Frost 2018). The type locality for *O. centralis* is Floresta Nacional de Silvânia (FLONA) in the state of Goiás, Brazil.

Ololygon centralis is the only species of the genus known to possess a macrogland; however, there has been no research on the morphology or the products of this gland. Therefore, herein we describe the inguinal gland of *O. centralis* using light and electron microscopy.

MATERIAL AND METHODS

Adult individuals of *O. centralis* were collected at night at the reproductive site when they were in reproductive activity. The specimens of *O. centralis* were collected at the type-locality (Floresta Nacional de Silvânia, Silvânia, GO, 16°38'04.4"S, 48°39'31.2"W) and transported to the campus of the Universidade Federal de Goiás (UFG, Goiânia, GO, Brazil). The specimens were euthanized with a topical application of lidocaine and the inguinal skin dissected and processed for microscopy. The specimens were deposited at the Zoology Collection of the Universidade Federal de Goiás (ZUFG) (Appendix 1). This study was approved by UFG's ethics committee (#109/14), according to federal regulations (CEUA 2018).

Inguinal skin samples from the male and female specimens were fixed in methacarn (1:3:6, acetic acid, chloroform, and methanol), dehydrated and embedded in paraffin. Histological sections (5 µm) were stained with Harris hematoxylin for one minute and aqueous eosin for approximately four minutes (HE) for general morphological analysis (modified from Suvarna et al. 2018).

Some skin samples were fixed in buffered 4% paraformaldehyde, dehydrated in alcoholic series and embedded in glycol methacrylate resin (Historesin®, Leica). To determine the chemical nature of the secretion of the glands, three micrometer thick sections were cut on a Leica RM2245 microtome and submitted to the histochemical staining procedures. Photomicrographs were taken with an Olympus BX-41 photomicroscope and morphometric analysis done with IMAGE PRO PLUS software. At least ten aleatory regions of each structure were measured, and the mean and the standard error calculated.

Periodic acid-Schiff (PAS). This reaction was performed to investigate the presence of basic glycoprotein in the glandular secretion. Sections were treated with 1% periodic acid for 20 minutes, rinsed in distilled water and then covered with Schiff's reagent for five minutes. The sections were then rinsed in running water for 10 minutes and then counterstained with Harris hematoxylin for approximately one minute (modified from Pearse 1960).

Nile blue. This procedure was performed to investigate the presence of melanin in skin layers. Some sections were stained with sulfuric Nile blue [0.05% Nile blue in 1% aqueous sulfuric acid solution (w/v)] for 20 minutes at room temperature and then rinsed for 20 minutes in running water. Other sections were treated with 0.25% potassium permanganate for 45 minutes followed by 5% oxalic acid for eight minutes for melanin bleaching, followed by staining with sulfuric Nile blue (modified from Pearse 1960).

Toluidine Blue. This procedure was performed for metachromatic detection of acid components. Sections were stained in toluidine blue solution [1% toluidine blue PA; 1% sodium tetraborate (w/w) in distilled water] for one minute and then rinsed in running water.

Ponceau Xylidine. This method was performed to investigate the presence of basic protein in glandular secretion. Sections were stained in Ponceau Xylidine solution [0.1% Ponceau Xylidine in 2.5% acetic acid (w/v)] for 20 minutes at room temperature and then rinsed in distilled water (modified from Suvarna et al. 2018).

Bromophenol Blue. This procedure was performed to determine the approximated pH of glandular secretion. Sections were stained in Bromophenol blue solution [0.5% Bromophenol blue ACS, 10% mercuric chloride in 2% acetic acid (w/w/v)] for two hours at room temperature, rinsed in 0.5% acetic acid and then rinsed briefly in tertiary-butyl alcohol (modified from Pearse 1960).

Alcian Blue 8GS pH 2.5. This procedure was performed to detect the presence of acid mucopolysaccharides in glandular secretion. Sections were stained in Alcian Blue at pH 2.5 [1% alcian blue 8GS in 3% acetic acid (w/v)] for 30 minutes at room temperature and then rinsed for 10 minutes in running water (modified from Junqueira and Junqueira 1983).

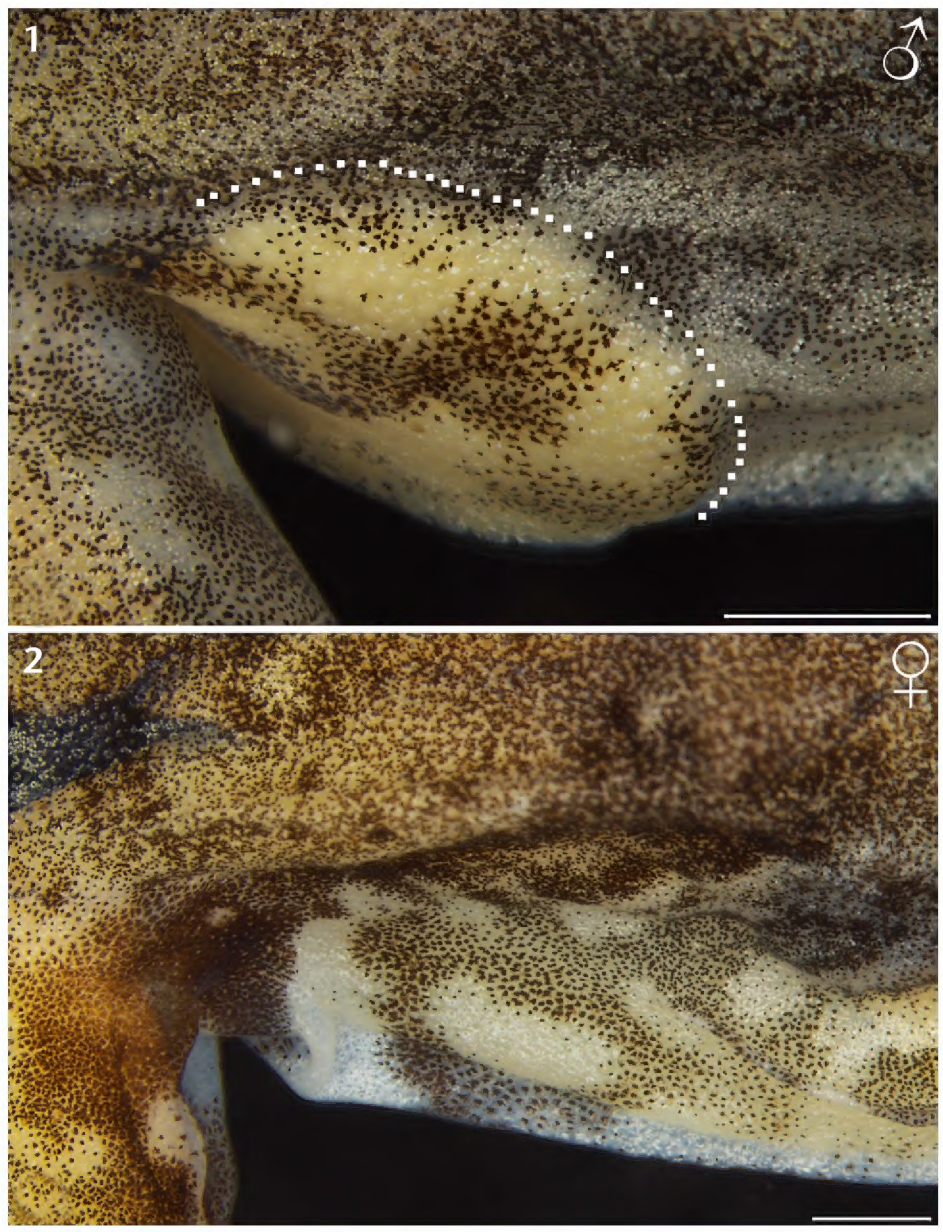
Alcian Blue 8GS pH 1.0. This procedure was performed to detect the presence of strongly sulfated mucosubstances in

glandular secretion. Sections were stained in Alcian Blue at pH 1.0 [1% Alcian Blue 8GS in Hydrochloric acid 0.1N (w/v)] for 30 minutes at room temperature and then dried with a filter paper (modified from Junqueira and Junqueira 1983).

For TEM, samples were fixed with 2.5% glutaraldehyde and 0.2% picric acid solution in 0.1M cacodylate buffer, pH 7.2, with 2% sucrose. The material was post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in an acetone series and embedded in epoxy resin. Ultrathin sections were stained with 2% uranyl acetate and 0.2% lead citrate and analyzed using a JEOL, JEM 2100 Transmission Electron Microscope under 80kV.

RESULTS

Males of *O. centralis* possess a round gland in the inguinal region that varies in size from approximately two to three millimeters in diameter (n = 8). Females have no gland structure, despite them having the same pigmentation pattern in the inguinal region (Figs 1–2). Observation under magnification revealed that the gland is composed of many round yellowish-colored dots (Fig. 1).



Figures 1–2. Photographs of the lateral sides of a *O. centralis* male (1) and female (2). The dashed line marks the limit of the inguinal gland in males that are absent in females. Scale bars: 1 mm.

Histological sections of the skin around four male inguinal glands revealed the presence of a stratified epithelial layer of approximately 14.5 (±1.8) µm thick supported by 30 (±12) µm of the dermis (Fig. 3). The dermis is divided into spongious dermis, where a layer of melanocytes is usually present, and dense dermis, with an abundance of collagen fibers. Roundish mucous glands of approximately 31 (±4) µm in diameter were observed in the spongious dermis (Fig. 3). In females, the entire region equivalent to the inguinal gland in males has the same histological characteristics of the skin described above, with no specialized structure (not shown).

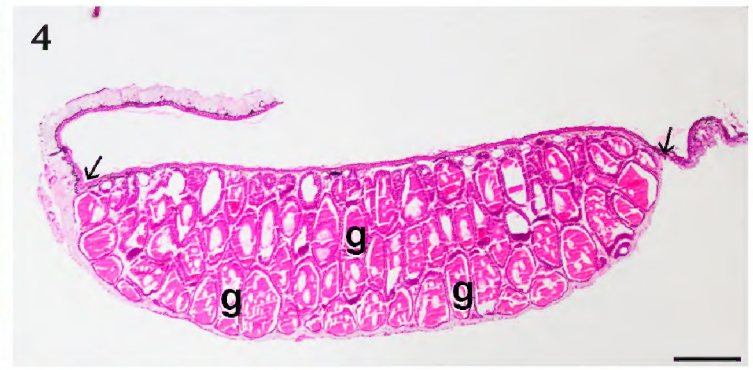
Histological sections of male inguinal glands revealed many syncytial serous glands, of more than 500 (±7.5) µm in height, approximately 78 (±17) µm in width and filled with colloidal eosinophilic secretion (Fig. 4). The syncytial glands are in the enlarged spongious dermis. Among the syncytial glands are roundish mucous glands near the epidermis, some conjunctive cells, blood vessels and loose extracellular material (Figs 5–7, 10). There is a short duct connected to the secretory portion that passes through the epidermis (Figs 5, 6). The central portion of the syncytium is filled with colloidal eosinophilic secretion (Figs 5, 6). This syncytium has basal nuclei, with irregular outline (Figs 13, 18), and agglomerations of colloidal material in the cytoplasm (Figs 8, 11–15). The colloidal secretion is electron dense and is also present in the cortex of the syncytium interspaced with cytoplasmic organelles (Figs 11–15). The cortex region of the syncytium, near the nuclei, is rich in rough endoplasmic reticulum and free ribosomes (Figs 12, 13). A discontinuous layer of myoepithelial cells is present external to the syncytial glands (Figs 5, 6, 11, 16, 17). There is a layer of extracellular matrix with thin collagen fibrils separating the myoepithelial cells of neighbor syncytia (Figs 16, 17).

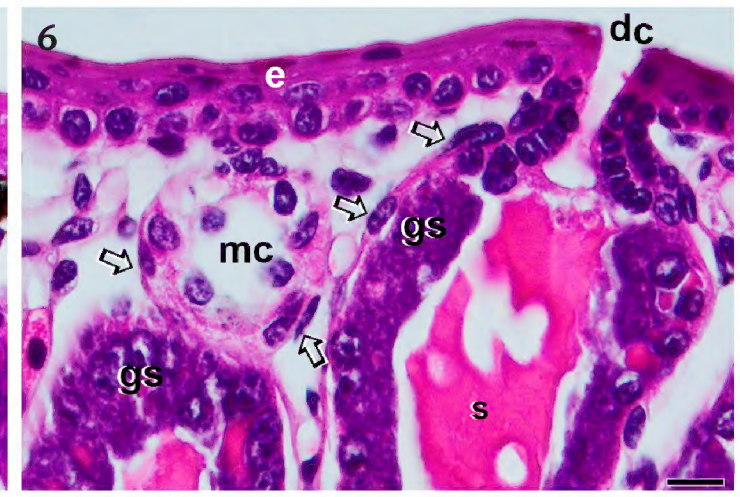
The roundish mucous glands comprise few cells (1–3) with cytoplasm that reacted positively to PAS (Fig. 7). No positive reaction was observed in the lumen of the mucous gland (Fig. 7), which appeared to be empty. The results of the histochemistry tests of the gland secretion of the serous syncytium are summarized in Table 1 and suggest that their secretion is composed mainly of basic proteins, with no, or very few, glycosylated portions. It is important to note that despite the pale blue color of the secretion due to Toluidine Blue, the cytoplasm

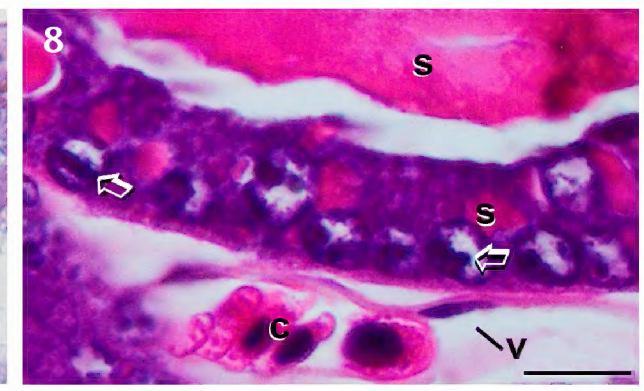
Table 1. Summary of the histochemistry tests used to study male serous inguinal glands of *Ololygon centralis*.

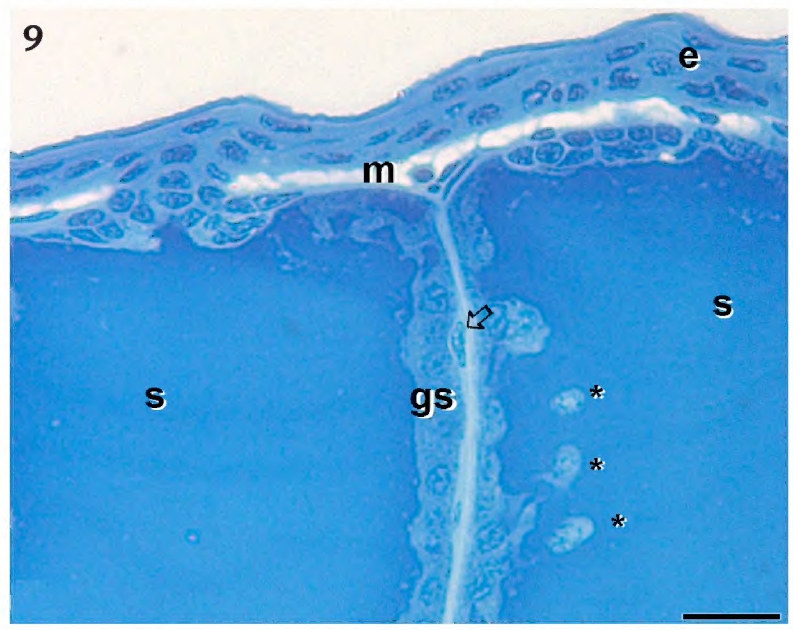
Technique	Results (gland secretion)	Significance
Xylidine Ponceau	Positive*	Presence of basic amino acid groups
Toluidine Blue	Pale	Absence of acid-negative groups
PAS	Negative	Absence of neutral sugar residues
Bromophenol Blue	Violet*	pH > 4.6
Alcian Blue pH 2.5	Negative*	Absence of mucopolysaccharides
Alcian Blue pH 1.0	Negative*	Absence of sulfated mucosubstances

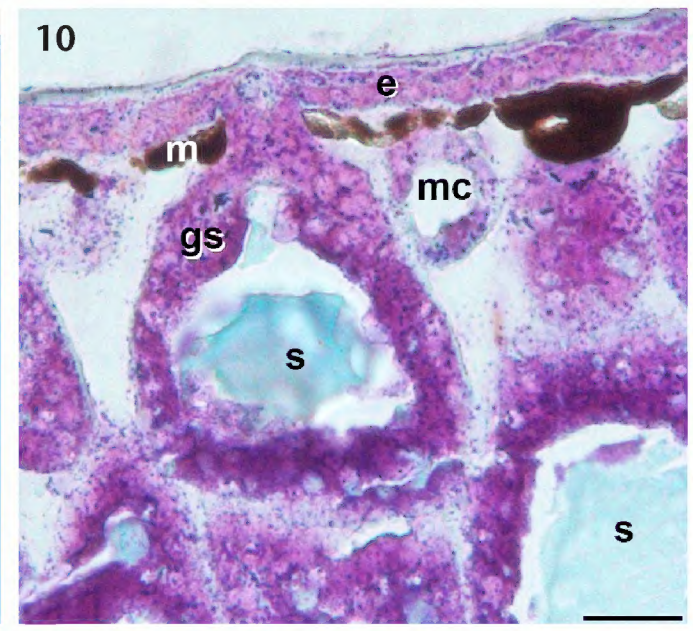
*Data not shown.



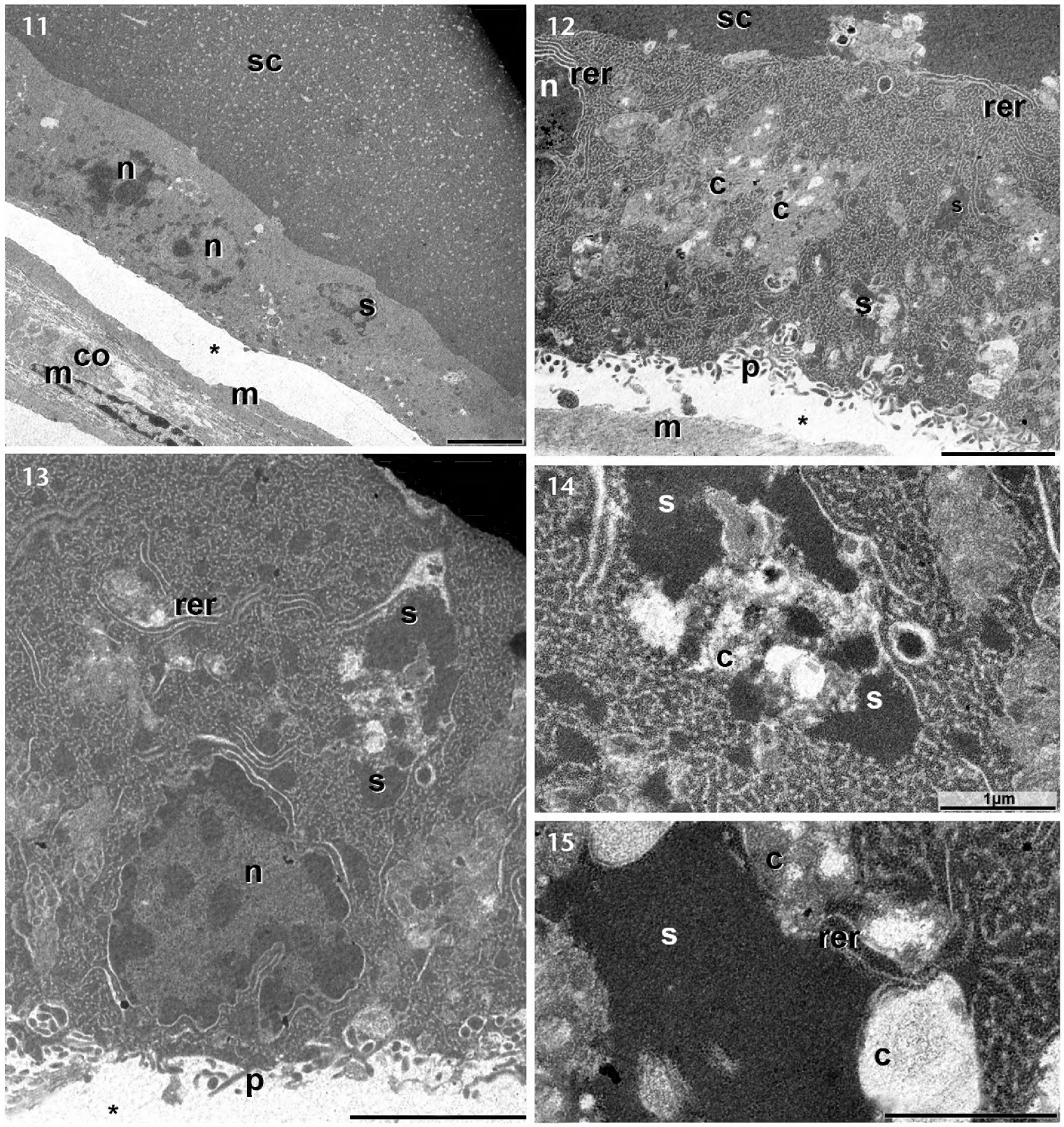








Figures 3–10. Photomicrographs of histological sections of the male inguinal gland region of *O. centralis*. (3–6, 8) Histological sections stained with HE. 3) Section of skin from the peripherical region of the inguinal gland. Notice that only mucous glands are present. 4) Low magnification micrograph showing the presence of many syncytial glands (g), with arrows indicating the lateral limits of the inguinal gland. (4–6) Major magnifications of the glandular apical portion, with many melanocytes (m), mucous glands (mc) and myoepithelial cells (open arrows). Note the glandular ducts (dc). 7) Histological section submitted to PAS reaction. Notice that only some cells of the mucous glands (mc) exhibit a positive reaction (arrowheads). (8) Major magnification of the lateral base portion of the syncytium, with colloidal secretion (s) in syncytium cytoplasm. Note also a blood vessel in the connective tissue. (9) Methacrylate section treated with potassium permanganate and oxalic acid and stained with Nile blue. Notice the bleached melanocytes (m) and some syncytial cytoplasmic projections (*) through the glandular secretion (s). (10) Methacrylate section stained with toluidine blue. Notice the pale blue color of the secretion suggesting it is alkaline, contrasting with the dark blue color of the glandular syncytium (gs). (e) epidermis; (d) dermis; (black open arrow) myoepithelial cells; (c) blood cells. Scale bars: 5, 6, 8 = 10 µm, 3, 7, 9, 10 = 20 µm; 4 = 200 µm.



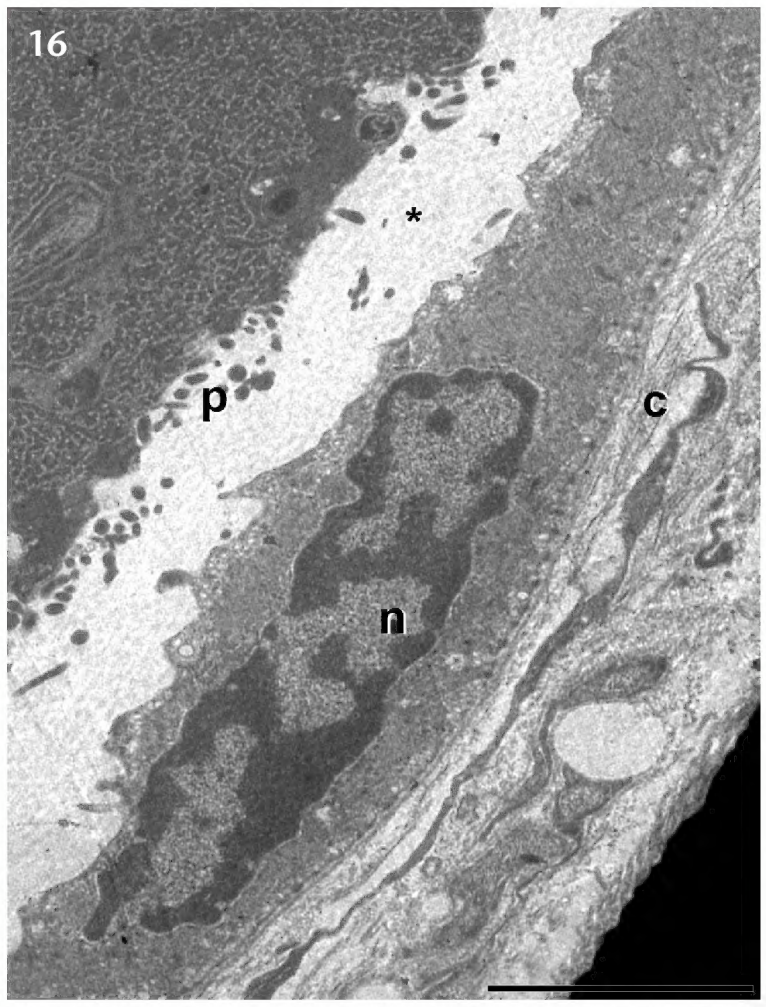
Figures 11– 15. Electron micrographs of the serous glands of the inguinal region. (11) Low magnification of the secretory syncytium with two visible nuclei (n) and also a sizeable cytoplasmic secretion aggregate (s). Notice the syncytium center (sc) filled with electron dense secretion and also the clear space (*) between syncytium basis and myoepithelial cells (m). Around the myoepithelial cells are some collagen fibrils (co). (12–13) Medium magnification of syncytium, where it is possible to notice some cytoplasmic secretion aggregate (s) and some regions of the cytoplasm with medium electron density (c). (14–15) Major magnifications of two large cytoplasmic secretion aggregate, with mixed portions of electron dense secretion (s) with medium electron density cytoplasm (c). (p) basal digitiform projections; (rer) rough endoplasmic reticulum. Sacale bars: 14, 15 = 1 µm, 12, 13 = 3 µm, 11 = 5 µm.

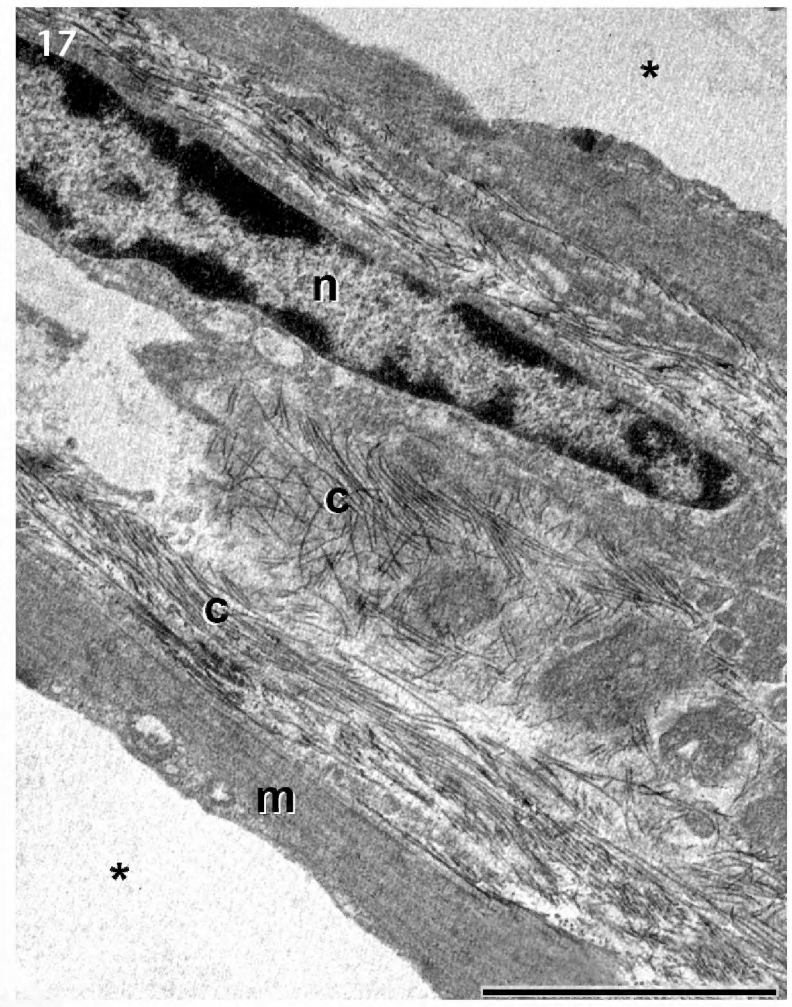
of the syncytium is dark blue/purple (Fig. 10). The potassium permanganate bleaching test confirmed the presence of melanin in the melanocyte layer below the epidermis (Fig. 9).

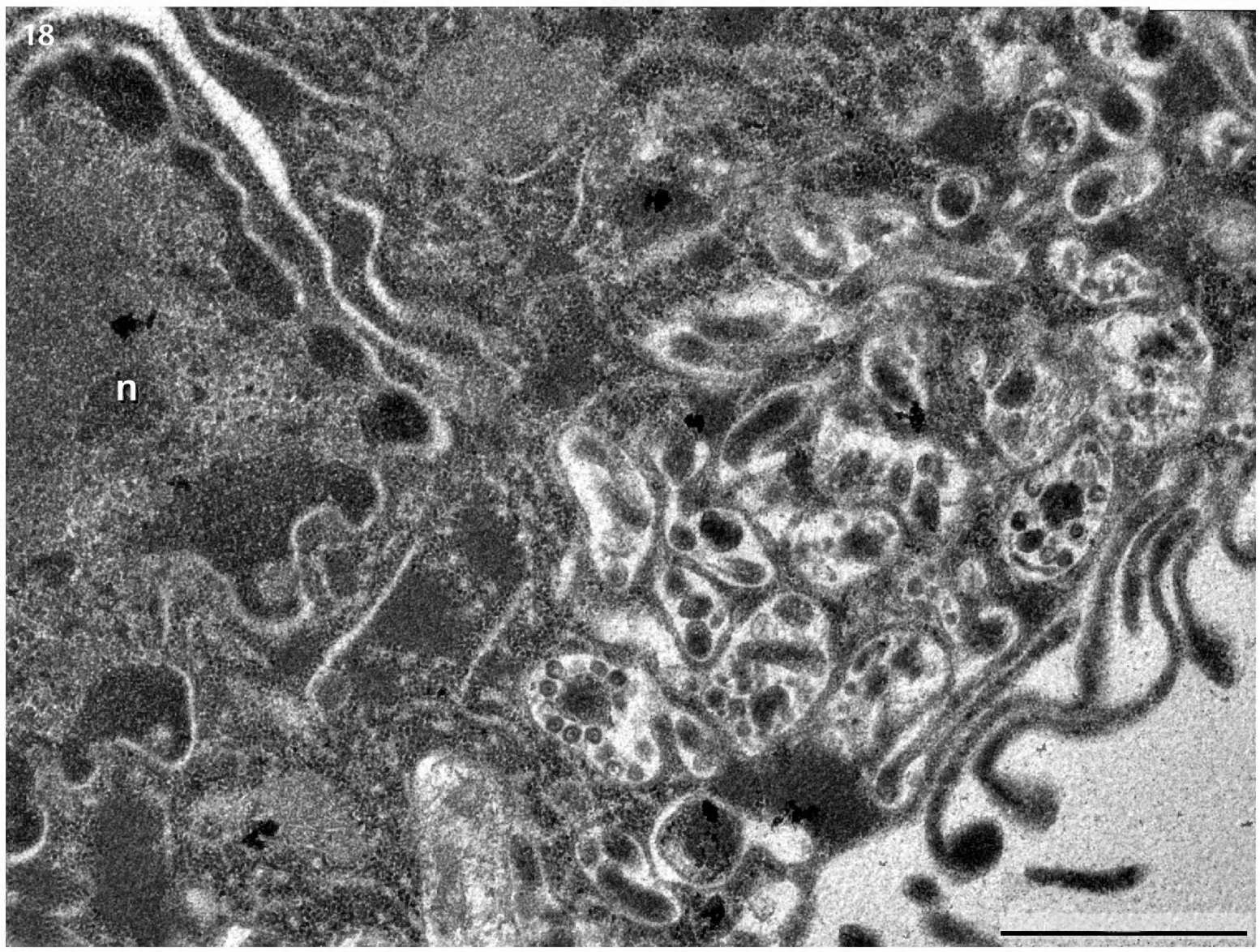
The base of the syncytium has many digitiform projections extending into the intercellular space among these cells and the myoepithelial cells (Figs 11–13, 16, 18). This space is probably an artifact of fixation. The delicate labyrinth formed by the projections does not exhibit many adhesive structures, which probably contributed to cell detachment (Fig. 18).

DISCUSSION

Males of *O. centralis* have quite unusual glands in the skin of the inguinal region. These macroglands are comprised mostly of large syncytial units. They differ from those of the more than 40 species that have been studied, in which serous skin glands comprise a syncytium with a central region constituted by a heterogeneous mixture of cell fragments (Delfino et al. 2001, Terreni et al. 2002, Alvarez et al. 2005, Lenzi-Matos et al. 2005,







Figures 16–18. (16) The basal portion of the syncytium with digitiform projections (p) and the clear space (*) between them and the myoepithelial cells. Notice the myoepithelial cells nuclei (n) and the collagen fibrils. (17) Detail of the connective tissue between two neighbor alveoli, with myoepithelial cells (m) and collagen fibrils (c). (18) The basal portion of a syncytium with intricate projection labyrinth. Notice the syncytium nucleus with irregular outline (n). Scale bars: 18 = 1 µm, 16, 17 = 3 µm.

Felsemburgh et al. 2007, 2009, Rigolo et al. 2008, Gonçalves and Brito-Gitirana 2008, Jared et al. 2009, Silva et al. 2017). An exception is *Odontophrynus americanus* Duméril & Bibron, 1841, which has typical mucous and granular glands, but also a third tubule-alveolar gland with the same morphological and histochemical characteristics as observed in *O. centralis* (Felsemburgh et al. 2007). These glands, however, are spread throughout the body instead of being concentrated in a specific region (Felsemburgh et al. 2007).

One species of *Scinax* (*S. hayii*) and two species of *Ololygon* (*O. agrensis*, *O. flavoguttata*) have serous skin glands with cell fragments interspaced by colloidal material in the "lumen" (Silva et al. 2017). These glands, however, are also not organized as macroglands.

Few species of frogs have had sexual dimorphic glands histochemically analyzed. The sexually dimorphic glands of *B. punctata* differ from those of *O. centralis*, by not being organized as macroglands and their secretion is formed of cell fragments (Brunetti et al. 2012). Males of *Cycloramphus fuliginosus* Tschudi, 1838, have inguinal macroglands (Gonçalves and Brito-Gitirana 2008) that are similar to those observed in *O. centralis*, and which produce an acidophilic serous secretion. Gonçalves and Brito-Gitirana (2008) suggested two possible functions for these glands: i) since they are observed only in males of *C. fuliginosus*, they may be involved in the production of aquatic sexual pheromones; or ii) the production of antimicrobial substances to protect ova. Unlike *C. fuliginosus*, males of *O. centralis* males do not attract females in an aquatic environment, but instead, embrace females on dry ground and then enter the water puddles (personal observation).

Most known amphibian sexual pheromones are dispersed in water (Wabnitz et al. 1999, Kikuyama et al. 2002, Houck 2009, Belanger and Corkum 2009). However, Belanger and Corkum (2009) argued that the olfactory organs of anurans could detect pheromones spread via water and air. It has been shown that male frogs of the family Mantellidae from Madagascar can attract specific females by volatile pheromones (Poth et al. 2012). Another hypothesis is that *O. centralis* males have a novel way of applying pheromones that involves delivering it directly to the female during amplexus, as is done by some salamander species during courtship (Houck 2009). These observations reinforce the possibility of that the inguinal glands of *O. centralis* function in secreting pheromones. This hypothesis, however, has yet to be experimentally tested.

Regardless of the function(s) of the inguinal gland of *O. centralis*, the confirmation of a sexually dimorphic gland in this species raises several questions and possibilities to be tested in future research. Surveying for similar glands among other species of the same genus, chemical analysis of the glandular contents, and testing the effects of the male secretion on female behavior are all examples of research that would shed more light on the function of this gland.

The cytoplasm of the syncytium of the serous gland of *O. centralis* is electron dense and possesses secretion aggregates of various sizes. The larger aggregates seem to be formed by the function of smaller aggregates and have an irregular outline and regions of variable electron density, suggesting that portions of cytoplasm are inside the secretion. These two characteristics suggest that there is no surrounding membrane. We believe that the electron dense (colloidal) secretion is produced by free polyribosomes, which differs from the idea proposed by Delfino et al. (2001). These authors described the maturation process occurring in vesicles from Golgi apparatus in serous glands of many frog species. Nonetheless, the morphological characteristics of the secretion in that study differ from those described in the present study, reinforcing the unusual characteristics of glands of *O. centralis*.

The intense Toluidine Blue coloration in the syncytium cytoplasm of the serous glands of *O. centralis* suggests it is rich in ribosomes. Although Toluidine Blue is not a specific stain, it interacts with anionic or polyanionic components (i.e., nucleic acids and glycosaminoglycans). Since the PAS and Alcian Blue tests do not suggest the presence of glycosaminoglycans, and the Bromophenol Blue test suggests a basic pH for the colloid, the intense blue/purple coloration of the syncytium cytoplasm indicates ribosome richness.

The basal portion of the syncytium, with an intricate labyrinth of digitiform projections, suggests intense traffic of substances between the syncytium and the connective tissue. Furthermore, the presence of myoepithelial cells indicates that they may be involved with secretion ejection.

Located between the serous glands of the inguinal macroglands of *O. centralis* are round mucous glands. These glands are very similar to the mucous glands reported for other anuran species (Lenzi-Matos et al. 2005, Alvarez et al. 2005, Rigolo et al. 2008, Jared et al. 2009, Felsemburgh et al. 2009, Brunetti et al. 2012), with round alveoli, possessing by PAS-positive epithelial cells. These glands are probably responsible for maintaining skin moisture.

Future studies must be performed to confirm the hypothesis of pheromone production by the inguinal glands of *O. centralis*. Validating this hypothesis would help to understand the complex mating behavior of this species, and perhaps change conservation strategies directed at them.

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Appendix 1. Information about specimens used in the study.

Voucher	Sex	SVL[1] (mm)	Stereomicroscopy	Light Microscopy	Electron Microscopy
ZUFG9287	F	24.60		Hematoxylin-Eosin	
ZUFG9292	F	24.80		Hematoxylin-Eosin	
ZUFG10377	F	24.86	X		
ZUFG9288	M	21.00		Histochemistry	
ZUFG9289	M	21.40		Histochemistry	
ZUFG10374	M	20.32	X		
Lost specimen 1*	M	20.00		Hematoxylin-Eosin	X
Lost specimen 2*	M	18.45		Hematoxylin-Eosin	X

[1]Snout-vent length.
*These specimens were destroyed during dissection and could not be deposited in the zoological collection.